A splicing isoform of GPR56 mediates microglial synaptic refinement via phosphatidylserine binding

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Review Timeline:

Editor: Karin Dumstrei

Transaction Report:

Dear Xianhua,

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

Both referees find the analysis interesting and I would like to invite you to submit a revised version. Referee #2 raises relative minor issues, while referee #1 more significant ones. I have carefully looked at the comments listed by referee #1 and while they are all reasonable I also find that not all issues have to be resolved for publication here. I have listed my comments below the specific issues raise by referee #1. Please respond to all the issues raised by referee #2.

I am happy to discuss the revisions further.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 9th Apr 2020.

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Referee #1:

In the present manuscript, Tao Li and Colleagues describe a novel role for the adhesion G protein-coupled receptor (aGPCR) ADGRG1/GPR56 expressed on microglial cells. By in vitro binding experiments and in vivo GPR56 full knockout or conditional knockout mice, they show that the alternatively spliced isoform of GPR56 (GPR56 S4) is required for microglia-mediated synapse elimination during postnatal life in the mouse. They also report that GPR56 S4 binds phosphatidylserine (PS) and that PS serves as an "eat-me" signal for synapse engulfment by microglia. Although the identification of PS as putative signal for synapse elimination and the clarification of the role of microglial GPR56 in the process would represent a major advancement in the field, the study lacks crucial experiments to support the drawn conclusion.

Figure 1 and page 5: The authors claim that PS is a new "eat-me" signal exposed at the synapse in the developing brain, based on the use of the PS marker PSVue and the anterograde tracer fluorescent cholera toxin B (CTB). Although the Authors show that some RGC inputs in the dLGN of WT mice are labeled by PSVue and that the extent of labeling reduces from P6 to P13 (thus paralleling the timing of microglia-mediated synapse engulfment) no evidence for synaptic labeling by PSVue is provided. The authors should stain brain sections with antibodies against either pre or

postsynaptic markers and show their colocalization with PSVue.

Karin: This issue is raised by both referees #1 and 2 - please address.

Fig. 1 C-D: It is known that in the dLNG microglial engulfment of synaptic elements peaks at P5 and is decreased at P10 (Schafer et al., 2012) and in fact the density of synaptic contacts is reduced in parallel. If CTB-488 positive structures represent synaptic terminals, a reduction of CTB-488 staining would be expected in P13 dLGN. The graph shows the % PS+ RGC inputs. Has this measure been normalized to the total number of CTB-488+ inputs?

Karin: please clarify

Fig. 1 E-F: synaptic engulfment should be demonstrated by showing synaptic markers internalized in microglial CD68-positive structures. Orthogonal sections should be shown and analyzed. The authors observe ~73% PS+ and 27% PS- RGC inputs within microglia. A parallel quantification of PVSue+ and RGC+ inputs inside and outside microglia is needed. Finally, 5-TAMRA does not prove that PSVue is specifically exposed at the synapse.

Karin: This point would be good to address and it looks like you have the tools on hand to address this issue. Let me know if there are any problems with doing the experiments

Fig. 2: this experiment lacks negative controls. The authors should perform the same experiments using different phospholipids (i.e. sphingomyelin; phosphatidylinositol; phosphatidylcholine; sulfatide; cardiolipin) in order to show that GPR56 specifically binds PS. Also, the authors use FITC-conjugated Annexin V as a positive control but do not show its binding to Ba/F3 cells (Fig. 1D). Finally, the use of primary neurons instead than Ba/F3 could be more relevant in the present context.

Karin: fine to use Ba/F3 cells no need to extend to primary cells. Would be good to test different phospholipids

Fig. 3: a functional characterization of microglia lacking GPR56 or GPR56 S4 would be essential. Is the general phagocytic activity of microglia impacted by the lack of either protein? Is the inflammatory profile changed?

Karin: if you have data on hand to address this point then please include it. If not then OK not to address this issue.

Fig. 3I: The authors analyzed GPR56 S4 transcript in microglia at P14 in WT mice. Which brain area is microglia isolated from? Does GPR56 expression vary at P5, when microglia are actively pruning synapse in the dLGN, with respect to P13?

Fig. 3 J-K: here the Authors, correctly, introduce the use of synaptic markers. However, defining the colocalizing Vglut1/homer1 structures as "synapse number" is not proper. The Authors should stick to the term "colocalizing Vglut1/homer1 puncta".

Karin: Please address

The quantitation of synaptic puncta engulfed by microglia in GPR56 KO and S4 is mandatory, in order to state that "GPR56 S4 isoform is crucial to developmental synaptic refinement". Also,

although the Authors report that the increased synapse density is not the result of altered RGC number, given that GPR56 is involved in neuronal development, a quantitation of neuronal arborization should be performed in GPR56 KO and S4, to exclude that the differences in Vglut1/homer1 positive puncta depends on neuron-autonomous factors, different from synaptic pruning. Also, based on the decrease in MBP in the corpus callosum, the possibility that demyelination might impact synaptic pruning by microglia should be at least discussed.

Karin: Please add quantification

Fig. 3K and L: The images in Fig. 3K show an apparent higher density of Vglut2+ puncta in Gpr56 null mice, while Homer1+ puncta seem to be increased in both GPR56 null and GPR56 S4. A quantification of Vglut2 or Homer1 puncta, separately, could be of interest to investigate the possibility that GPR56 may be involved in mediating predominantly the elimination of presynaptic or postsynaptic puncta.

Karin: If straight forward enough to do then please address this issue

Fig. S3 and Bennet et al., PNAS 2016, show that GPR56 transcript increases in microglia during development. The authors should discuss the meaning of this increase, since, according to their hypothesis, GPR56 is critical during the synaptic pruning period.

Karin: Please discuss

Fig. 4: while this figure is consistent with the concept that "Deleting microglial GPR56 results in excess synapses in the dorsolateral geniculate nucleus during postnatal development", again no evidence is presented that the increased synaptic density is the consequence of a defect in synaptic pruning. Some experiments would be crucial to address this possibility: qPCR should be performed to demonstrate that the higher synaptic protein expression does not result from increased synaptic protein transcription in mutant mice. Even more importantly, analysis of synaptic engulfment by microglia should be performed by quantifying the synaptic markers internalized in microglial CD68-positive structures.

Karin: I realise that this experiment is not straight forward but would be good to address

Fig. 4 C and D: A quantification of Vglut2+ or Homer1+ puncta, separately, could be of interest (see above).

Karin: see my comments to related point above

Fig. 4 E-H: Quantification by structured illumination microscopy (SIM) was performed in P8 mice brains, while WB analysis of CTR and CKO mice was performed at P30. The authors should add WB quantification of Vglut2 in the dLGN also at P8. Also, WB quantification of postsynaptic markers should be added, together with qPCR analyses for both pre and postsynaptic protein mRNAs.

Karin: please address

Page 14: "Considering that PS binds GPR56 and flags synapses for removal by microglia" This sentence should be modified. The authors only showed that PSVue+ puncta colocalize with CTB-488. They did not perform staining with synaptic markers and omitted to do additional controls (see comments to Fig.1).

Karin: please address

Fig. 5: this figure should be removed. A convincing demonstration that "microglial GPR56 regulates hippocampal synaptic development in a circuit-dependent manner" would require significantly deeper analyses (characterization of GPR56 expression in different hippocampal regions, quantitation of microglial engulfed synaptic material, Western Blotting, qPCR and so on).

Karin: I would suggest to leave the figure in as I do think it adds insight. make sure that the conclusions from this figure are not overstated

Fig. 7A-B: Please, see the comments above (Figure 1 C-D)

Fig. 8: the scheme provided is far ahead of the actual results reported in this manuscript.

Karin: I am OK with the figure and find it helpful. I would leave in.

Minor:

Page 6: Please provide the expanded definition for NTF. The figure legends 2 A and B should explain better how the authors engineered recombinant proteins.

Fig. 4 A and B. Which area were analyzed? What is the mice's age? Please, specify this in the text and fig. legends.

Minor: Pag. 11: Please provide the expanded definition for Brn3a+

Referee #2:

In this well-written and elegant study, Li and colleagues seek out to determine the function of microglial GPR56 in normal development. Having previously demonstrated the functions of GPR56 in cortical development and oligodendrocyte maturation, the authors ask whether microglial GPR56 is involved in normal synaptic pruning. They determined that a specific isoform of GPR56 (S4), containing only a GAIN domain, acts as a receptor for phosphatidylserine (PS+) which is required for most microglia-mediated phagocytosis of PS+ RGC synapses during retinogeniculate refinement. This isoform is preferentially expressed by microglia. The authors also link the defect in synapse pruning to functional consequences, and show that GPR56-mediated synaptic pruning is also at play in the developing hippocampus. Overall, this is a topic of wide interest to the readership of this journal, given growing interest in the function of microglia in development, health, and disease, and that this manuscript beautifully demonstrates the importance of splice variants in cell type specific functions of a G-protein coupled receptor expressed by multiple cell types. This reviewer has only a few concerns that if address would improve the impact of the study:
Using PSVue to label PS presynaptic elements or PS on BA/F3 cells, the authors show that the GAIN domain of GPR56 binds PS. For the invivo synaptic pruning experiments, the authors use the widely used anterograde tracer cholera toxin B to show PS+ engulfment. Could the authors address if there are any confounders about CTB binding lipid rafts which presumably PSVue may also bind?

For example, to show that PS+ terminals are indeed presynaptic phagocytosed elements, combining analysis of PS+ Homer+ elements within microglia?
It would be helpful to include a diagram as in Fig3A with the GPR56 NULL mouse for comparison. As the stated justification to use CX3CR1-Cre, this reviewer would recommend authors not necessarily use Zhao et al 2019 as proof that it is microglia specific, since the reasoning is less likely non-specific recombination of the reporter but rather different sensitivity of reporters; would instead recommend that authors emphasize their efforts to show microglia specific knockout. Speaking of: Great work on using the RNAscope to show loss of GPR56 in microglia. This is not, however, showing microglia-specificity, as an analysis of # non-microglial cells expressing GPR56 would be a more valid approach. Unless looking at recombination, however, would rephrase explanation that their approach unlikely non-specifically targeting cells in the retinogeniculate system given that GPR56 levels in non-microglial cells are comparable.
Is the % increase in synaptic number observed for CKO for the various methods (Homer/VGlut1, SIM) comparable?
In the abstract, the authors state that developmental synaptic remodeling defects lead to neurodevelopmental disorders. This struck me as a bit absolute - not all neurodevelopmental are caused by synaptic pruning defects, and it is largely an implication that this process underlies neurodevelopmental disorders.. Rather than direct lines of proof. The authors write with a more balanced voice in the Concluding remarks and I would recommend they slightly edit the language in the abstract to respect this nuance.

We are grateful for the constructive comments from the reviewers. In response to the reviewers' comments, we have performed additional experiments. Below, please find our point-by-point responses to the reviewers' comments. In addition, we have revised the manuscript in accordance with these suggestions.

Reviewer #1

1. *Figure 1 and page 5: The authors claim that PS is a new "eat-me" signal exposed at the synapse in the developing brain, based on the use of the PS marker PSVue and the anterograde tracer fluorescent cholera toxin B (CTB). Although the Authors show that some RGC inputs in the dLGN of WT mice are labeled by PSVue and that the extent of labeling reduces from P6 to P13 (thus paralleling the timing of microglia-mediated synapse engulfment) no evidence for synaptic labeling by PSVue is provided. The authors should stain brain sections with antibodies against either pre or postsynaptic markers and show their colocalization with PSVue.*

Response: We appreciate the suggestion. We have performed the immunostaining of vGlut2 and Homer1 in P6 dLGN, and demonstrated that vGlut2 and Homer1 are colocalized with PSVue (Please see new Fig. 1E and F, Page 5, line 19-23).

2. *Fig. 1 C-D: It is known that in the dLGN microglial engulfment of synaptic elements peaks at P5 and is decreased at P10 (Schafer et al., 2012) and in fact the density of synaptic contacts is reduced in parallel. If CTB-488 positive structures represent synaptic terminals, a reduction of CTB-488 staining would be expected in P13 dLGN. The graph shows the % PS+ RGC inputs. Has this measure been normalized to the total number of CTB-488+ inputs?*

Response: The % PS$^+$ RGC inputs was normalized and calculated as the number of PS$^+$ RGC inputs in one field over the number of total RGC inputs in the same field. (Please see Figure legend 1(D), Page 35, line 9).

3. *Fig. 1 E-F: synaptic engulfment should be demonstrated by showing synaptic markers internalized in microglial CD68-positive structures. Orthogonal sections should be shown and analyzed. The authors observe ~73% PS+ and 27% PS- RGC inputs within microglia. A parallel quantification of PVSue+ and RGC+ inputs inside and outside microglia is needed. Finally, 5-TAMRA does not prove that PSVue is specifically exposed at the synapse.*

Response: We are thankful for the suggestions. We have replaced the word "synapse" with "RGC synaptic inputs" in the text (Please see Page 4-6). We performed Iba1/CD68/CTB/PSVue co-staining, and showed PSVue$^+$ RGC inputs colocalized with CD68 and Iba1 (Please see new Fig. 1G, and page 6, line 13-15). We also carried out Iba1/CD68/vGlut2/PSVue co-staining, and showed PSVue$^+$/vGlut2$^+$ retinogeniculate synapses colocalized with CD68 and Iba1 (Please see new Fig. 1H, and Page 6, line 15). We also performed parallel quantification of PSVue$^+$ RGC inputs inside as well as outside of microglia (Fig. 1I and J, Page 6. line 16-19).

With regards to "*5-TAMRA does not prove that PSVue is specifically exposed at the synapse,*" we apologize for the confusion. We have revised and reemphasized that we used 5-TAMRA as a negative control and found that very little 5-TAMRA signal colocalized with RGC inputs and engulfed by microglia, which suggested that microglia don't engulf free fluorophore and the PSVue signals inside microglia were not free fluorophore (Page 6, line 6-11).

4. *Fig. 2: this experiment lacks negative controls. The authors should perform the same experiments using different phospholipids (i.e. sphingomyelin; phosphatidylinositol; phosphatidylcholine; sulfatide; cardiolipin) in order to show that GPR56 specifically binds PS. Also, the authors use FITC-conjugated Annexin V as a positive control but do not show its binding to Ba/F3 cells (Fig. 1D). Finally, the use of primary neurons instead than Ba/F3 could be more relevant in the present context.*

Response: We appreciate the suggestions. Unfortunately, it is not possible to perform FACS analysis for other phospholipids due to the lack of fluorophore-tagged reagents. Instead, we performed a protein-lipid overlay experiment using Membrane Lipid Strips and included the data in this revised manuscript (Please see Page 8, line 3-14).

Given that FITC-conjugated Annexin V was in different channel as AF647 and cannot be combined in Fig. C. We have since include the data in Appendix Fig.S2.

Indeed, primary neurons would be more relevant. However, we do not have the ability to reliably induce primary neurons undergo PS externalization. Furthermore, our goal is to demonstrate GPR56 can binds phosphatidylserine on live cells. In this regard, Ba/F3 cell line is well established cell-based model system (Gyobu et al., 2017; Miyanishi et al., 2007; Suzuki et al., 2010).

5. *Fig. 3: a functional characterization of microglia lacking GPR56 or GPR56 S4 would be essential. Is the general phagocytic activity of microglia impacted by the lack of either protein? Is the inflammatory profile changed?*

Response: Our *in vivo* engulfment assay showed that microglial GPR56 conditional ko (CKO) mice exhibited impaired phagocytosis of RGC inputs (See Fig. EV 5C and D). Interestingly, microglia lacking GPR56 didn't show significant defects in general cellular properties, like cellular density, %CD68, morphology (See Fig. EV 3C-J).

6. *Fig. 3I: The authors analyzed GPR56 S4 transcript in microglia at P14 in WT mice. Which brain area is microglia isolated from? Does GPR56 expression vary at P5, when microglia are actively pruning synapse in the dLGN, with respect to P13?*

Response: We thank the reviewer for the comments. We included experimental details in the method section in this revised manuscript. "Microglia were isolated from whole brains without cerebellum" (Page 25, line 11). Furthermore, we performed qPCR using P5 microglia, and showed that a steady increased of the transcript level from P5 to P14, which is consistent with previous reports (Fig. 3I, Appendix Fig. S1 and Bennet et al., PNAS 2016).

7. *Fig. 3 J-K: here the Authors, correctly, introduce the use of synaptic markers. However, defining the colocalizing Vglut1/homer1 structures as "synapse number" is not proper. The Authors should stick to the term "colocalizing Vglut1/homer1 puncta".*

Response: We agree with the reviewer's comment. In this revised manuscript, we specified colocalized vGlut2[+]/Homer1^{+} puncta as "retinogeniculate synapses" in the dLGN (Page 9, line 22), and vGlut1[+]/Homer1^{+} puncta as "corticogeniculate synapses" in the dLGN (Page 13, line 9).

8. *Fig. 3: The quantitation of synaptic puncta engulfed by microglia in GPR56 KO and S4 is mandatory, in order to state that "GPR56 S4 isoform is crucial to developmental synaptic refinement".*

Response: We thank the reviewer for the comment and have since performed the experiments and included our new data (Fig. 3M-O).

9. *Fig. 3: Also, although the Authors report that the increased synapse density is not the result of altered RGC number, given that GPR56 is involved in neuronal development, a quantitation of neuronal arborization should be performed in GPR56 KO and S4, to exclude that the differences in Vglut1/homer1 positive puncta depends on neuron-autonomous factors, different from synaptic pruning.*

Response: This request is reasonable in principle. Unfortunately, we found our inability to fulfill the request due to the following reasons:
1. Synapse number could alter without change in neuronal arborization. The synapses we analyzed are vGlut2$^+$ presynaptic inputs from RGCs. In early postnatal stage, the number of synapses decreases dramatically, but the average RGC arbor size and complexity remain largely unchanged (Hong et al., 2014). RGCs exhibit many *en passant* synapses (synapses on the stem of the RGC axons) that undergo elimination without changing the axonal structure (Hong et al., 2014).

2. Golgi staining will not work in this case, because it can't differentiate RGC arbors from dendritic arbors of local relay neurons and cortical neuronal arbors in the dLGN. Importantly, cortical inputs comprise ~90% synapses in dLGN.

3. It seems to us that generating RGC reporter line to visualize arbors as used in Hong et al., Eur J Neurosci 2019 would be an option. However, this will require up to 1-2 years of time to cross the reporter line into Gpr56 KO and S4 mutant background. Furthermore, there are over 20 different types of RGC cells, and different RGC classes exhibit distinct arborization patterns in the dLGN (Hong et al., 2019), and it is impossible for us to determine which type of RGC cells to study.

4. It is correct that GPR56 is expressed in first born neurons in the developing neocortex. However, it is largely absent in mature neurons. Furthermore, to address neuron-autonomous factors, we generated both microglial *Gpr56* constitutive conditional ko and microglial *Gpr56* tamoxifen-inducible ko mice, where neuronal *Gpr56* expression was not affected. We observed similar synapse defects as seen in *Gpr56* global ko mice, supporting that the synaptic chance was NOT due to "neuron-autonomous factors".

10. *Fig. 3: Also, based on the decrease in MBP in the corpus callosum, the possibility that demyelination might impact synaptic pruning by microglia should be at least discussed.*

Response: We thank the reviewer for the comment. We discussed it in the main text (please see Page 18, line 6-12).

11. *Fig. 3K and L: The images in Fig. 3K show an apparent higher density of Vglut2+ puncta in Gpr56 null mice, while Homer1+ puncta seem to be increased in both GPR56 null and GPR56 S4. A quantification of Vglut2 or Homer1 puncta, separately, could be of interest to investigate the possibility that GPR56 may be involved in mediating predominantly the elimination of presynaptic or postsynaptic puncta.*

Response: We quantified the densities of vGlut2$^+$ presynaptic inputs and homer1$^+$ postsynaptic signals, and only found increased vGlut2$^+$, but not homer1$^+$ in *Gpr56 null* (see new Appendix Figure S4, Page 10, line 1-2).

12. *Fig* S3 and Bennet et al., PNAS 2016, show that GPR56 transcript increases in microglia during development. The authors should discuss the meaning of this increase, since, according to their hypothesis, GPR56 is critical during the synaptic pruning period.

Response: We appreciate this comment. *Gpr56* transcript increases in microglia from embryonic stage and reaches a relatively high level between P3-P6, a period of active microglia-mediated synaptic pruning (Appendix Fig. S1, Page 7, line 1-2).

13. *Fig. 4: while this figure is consistent with the concept that "Deleting microglial GPR56 results in excess synapses in the dorsolateral geniculate nucleus during postnatal development", again no evidence is presented that the increased synaptic density is the consequence of a defect in synaptic pruning. Some experiments would be crucial to address this possibility: qPCR should be performed to demonstrate that the higher synaptic protein expression does not result from increased synaptic protein transcription in mutant mice. Even more importantly, analysis of synaptic engulfment by microglia should be performed by quantifying the synaptic markers internalized in microglial CD68-positive structures.*

Response: We appreciate this suggestion. However, we are constrained by technical limitations. Dorothy Schafer showed that most proteins engulfed by microglia will be quickly degraded once in lysosomes, which makes it unreliable to do synaptic engulfment analysis (Schafer et al., 2014). On the contrary, Alexa dye is more resistant to lysosomal hydrolases (Mukhopadhyay et al., 2010), making Alexa conjugated CTB as a robust dye to label engulfed material. Therefore, we performed our microglial engulfment assay using state of the art technology and showed a decreased engulfment in microglial GPR56 ko mice (Please see Fig. EV5C and D).

14. *Fig. 4 C and D: A quantification of Vglut2+ or Homer1+ puncta, separately, could be of interest (see above).*

Response: We included the quantification of Vglut2$^+$ or Homer1$^+$ puncta as a new Appendix Fig. S6.

15. *Fig. 3*: 4 E-H: Quantification by structured illumination microscopy (SIM) was performed in P8 mice brains, while WB analysis of CTR and CKO mice was performed at P30. The authors should add WB quantification of Vglut2 in the dLGN also at P8. Also, WB quantification of postsynaptic markers should be added, together with qPCR analyses for both pre and postsynaptic protein mRNAs.

Response: We performed the experiment as suggested and included the new data in this revised manuscript (Please see new Fig. 4 G-I, Page 12, line 6-9).

16. *Page 14: "Considering that PS binds GPR56 and flags synapses for removal by microglia" This sentence should be modified. The authors only showed that PSVue+ puncta colocalize with CTB-488. They did not perform staining with synaptic markers and omitted to do additional controls (see comments to Fig.1).*

Response: We appreciate the suggestion and modified the sentence as "Considering that PS binds GPR56 and flags RGC presynaptic inputs for removal by microglia" (Page 15, line 18).

17. *Fig. 5: this figure should be removed. A convincing demonstration that "microglial GPR56 regulates hippocampal synaptic development in a circuit-dependent manner" would require significantly deeper analyses (characterization of GPR56 expression in different hippocampal regions, quantitation of microglial engulfed synaptic material, Western Blotting, qPCR and so on).*

Response: In response to the reviewer's comment and editor's guidance, we have reworded our statement as "Microglial GPR56 regulates hippocampal synaptic development" (Page 13, line 14).

18. *Fig.* 7A-B: Please, see the comments above (Figure 1 C-D)

Response: As the same as Fig 1C-D, the measure was normalized to total number of RGC inputs.

19. *Fig. 8: the scheme provided is far ahead of the actual results reported in this manuscript.*

Response: We respectively disagree with this comment. Gratefully, we thank our editor Dr. Karin Dumstrei on her encouragement of including this figure.

20. *Page 6: Please provide the expanded definition for NTF. The figure legends 2 A and B should explain better how the authors engineered recombinant proteins.*

Response: We added the full name "N-terminal fragment" for NTF in the main text (Page 7, line 7). And in the Figure legend 2, we changed it to "(A) A schematic drawing of GPR56 protein structure, with a N-terminal fragment (NTF), a seven transmembrane domain (7-TM) and a C-terminal fragment (CTF). (B) A diagram shows the hFc tag was added to the c-terminal of GPR56-NTF (NTF-hFC) or GAIN domain (GAIN-hFc)." (Page 36, line 2-5)

21. *Fig. 4 A and B. Which area were analyzed? What is the mice's age? Please, specify this in the text and fig. legends.*

Response: We added the details of brain area and age in the main text: "We further performed RNAscope analysis for *Gpr56* in the prefrontal cortex of P30 mice" (Page 10, line 21-22). In the figure legends 4, we added "RNAscope was performed in the prefrontal cortex of P30 mice." (Page 39, line 5)

22. *Pag. 11: Please provide the expanded definition for Brn3a+*

Response: We added the definition for Bra3a+ in the main text. "brain-specific homeobox/POU domain protein 3A positive (Brn3a$^+$, a marker of RGC). (Page 12, line 10)

Reviewer #2:
1. *Using PSVue to label PS presynaptic elements or PS on BA/F3 cells, the authors show that the GAIN domain of GPR56 binds PS. For the in vivo synaptic pruning experiments, the authors use the widely used anterograde tracer cholera toxin B to show PS+ engulfment. Could the authors address if there are any confounders about CTB binding lipid rafts which presumably PSVue may also bind? For example, to show that PS+ terminals are indeed presynaptic phagocytosed elements, combining analysis of PS+ Homer+ elements within microglia?*

Response: We appreciated the reviewer for this thoughtful comment. To demonstrate the specificity of PSVue binding to synapses, we performed additional experiment, Iba1/CD68/vGlut2/PSVue co-staining, and showed PSVue$^+$/vGlut2$^+$ retinogeniculate synapses colocalized with CD68, and phagocyted by microglia (Please see new Fig. 1H, Page 6, line 15). Considering the fact that CTB binds lipid rafts, we

would expect to observe a similar colocalization between P6 and P13 if PSVue also binds to lipid rafts. However, we observed that PSVue only colocalized with ~10% CTB at P6 and 3% at P13.

2. *It would be helpful to include a diagram as in Fig3A with the GPR56 NULL mouse for comparison.*

Response: We thank the reviewer for the thoughtful suggestion and included a diagram in Fig 3A in this revised manuscript.

3. *As the stated justification to use CX3CR1-Cre, this reviewer would recommend authors not necessarily use Zhao et al 2019 as proof that it is microglia specific, since the reasoning is less likely non-specific recombination of the reporter but rather different sensitivity of reporters; would instead recommend that authors emphasize their efforts to show microglia specific knockout.*

Response: We thank the reviewer for the suggestion. We removed the paper of Zhao et al 2019 and included the discussion on our generation of the microglial *Gpr56* specific knockout mice. (Page 10, paragraph 3).

4. *Using the RNAscope to show loss of GPR56 in microglia… is not, however, showing microglia-specificity, as an analysis of # non-microglial cells expressing GPR56 would be a more valid approach. Unless looking at recombination, however, would rephrase explanation that their approach unlikely non-specifically targeting cells in the retinogeniculate system given that GPR56 levels in non-microglial cells are comparable.*

Response: We appreciate reviewer's suggestion. In this revised manuscript, we included a new Fig. 4B and rephrased our discussion (Page 10, line 21-23; Page 11, line 1-2).

5. *Is the % increase in synaptic number observed for CKO for the various methods (Homer/VGlut1, SIM) comparable?*

Response: The % of increase was different but comparable between SIM and confocal data. Specifically, we observed ~35% increase in CKO at P10 using traditional confocal imaging, and a ~50% increase in CKO at P8 using SIM. The small difference could result from age differences (P10 vs P8), and/or from method differences (traditional confocal vs SIM).

6. *In the abstract, the authors state that developmental synaptic remodeling defects lead to neurodevelopmental disorders. This struck me as a bit absolute - not all neurodevelopmental are caused by synaptic pruning defects, and it is largely an implication that this process underlies neurodevelopmental disorders.. Rather than direct lines of proof. The authors write with a more balanced voice in the Concluding remarks and I would recommend they slightly edit the language in the abstract to respect this nuance.*

Response: We appreciate the comments and have since reworded "Developmental synaptic remodeling is important for the formation of precise neural circuitry and its disruption has been linked to neurodevelopmental disorders such as autism and schizophrenia." (Page 2, line 1-3).

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Dear Xianhua,

Thanks for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referee #2 and as you can see from the comments below, the the referee appreciate the introduced changes and supports publication here. I am therefore pleased to accept the manuscript for publication here. Before sending you the formal accept letter there are just a few editorial comments to take care of.

- Keywords are missing

- Author contribution is missing for Tatsuhiro Koshi

- For the data availability section: Please state "This study includes no data deposited in external repositories"

- The appendix figure files need to be added together in one appendix that also includes the legends from the figures. This file should also have a ToC

- The movie file needs to be zipped together with its legend.

- We also include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by 400 high (pixels). You can also use something from the figures if that is easier.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data edited manuscript". Please take a look at the word file and the comments regarding the figure legends and respond to the issues. Please also resubmit the revised version with the marked changes - just makes it easier for me to see the changes.

That should be all. Let me know if you have any further questions.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

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- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Referee #2:

As this is a re-review, I will keep it brief:

The authors have sufficiently addressed this reviewer's concerns in their revised manuscript.

We are grateful for the constructive comments from the reviewers. In response to the reviewers' comments, we have performed additional experiments. Below, please find our point-by-point responses to the reviewers' comments. In addition, we have revised the manuscript in accordance with these suggestions.

Reviewer #1

1. *Figure 1 and page 5: The authors claim that PS is a new "eat-me" signal exposed at the synapse in the developing brain, based on the use of the PS marker PSVue and the anterograde tracer fluorescent cholera toxin B (CTB). Although the Authors show that some RGC inputs in the dLGN of WT mice are labeled by PSVue and that the extent of labeling reduces from P6 to P13 (thus paralleling the timing of microglia-mediated synapse engulfment) no evidence for synaptic labeling by PSVue is provided. The authors should stain brain sections with antibodies against either pre or postsynaptic markers and show their colocalization with PSVue.*

Response: We appreciate the suggestion. We have performed the immunostaining of vGlut2 and Homer1 in P6 dLGN, and demonstrated that vGlut2 and Homer1 are colocalized with PSVue (Please see new Fig. 1E and F, Page 5, line 19-23).

2. *Fig. 1 C-D: It is known that in the dLGN microglial engulfment of synaptic elements peaks at P5 and is decreased at P10 (Schafer et al., 2012) and in fact the density of synaptic contacts is reduced in parallel. If CTB-488 positive structures represent synaptic terminals, a reduction of CTB-488 staining would be expected in P13 dLGN. The graph shows the % PS+ RGC inputs. Has this measure been normalized to the total number of CTB-488+ inputs?*

Response: The % PS$^+$ RGC inputs was normalized and calculated as the number of PS$^+$ RGC inputs in one field over the number of total RGC inputs in the same field. (Please see Figure legend 1(D), Page 35, line 9).

3. *Fig. 1 E-F: synaptic engulfment should be demonstrated by showing synaptic markers internalized in microglial CD68-positive structures. Orthogonal sections should be shown and analyzed. The authors observe ~73% PS+ and 27% PS- RGC inputs within microglia. A parallel quantification of PVSue+ and RGC+ inputs inside and outside microglia is needed. Finally, 5-TAMRA does not prove that PSVue is specifically exposed at the synapse.*

Response: We are thankful for the suggestions. We have replaced the word "synapse" with "RGC synaptic inputs" in the text (Please see Page 4-6). We performed Iba1/CD68/CTB/PSVue co-staining, and showed PSVue$^+$ RGC inputs colocalized with CD68 and Iba1 (Please see new Fig. 1G, and page 6, line 13-15). We also carried out Iba1/CD68/vGlut2/PSVue co-staining, and showed PSVue$^+$/vGlut2$^+$ retinogeniculate synapses colocalized with CD68 and Iba1 (Please see new Fig. 1H, and Page 6, line 15). We also performed parallel quantification of PSVue$^+$ RGC inputs inside as well as outside of microglia (Fig. 1I and J, Page 6. line 16-19).

With regards to "*5-TAMRA does not prove that PSVue is specifically exposed at the synapse,*" we apologize for the confusion. We have revised and reemphasized that we used 5-TAMRA as a negative control and found that very little 5-TAMRA signal colocalized with RGC inputs and engulfed by microglia, which suggested that microglia don't engulf free fluorophore and the PSVue signals inside microglia were not free fluorophore (Page 6, line 6-11).

4. *Fig. 2: this experiment lacks negative controls. The authors should perform the same experiments using different phospholipids (i.e. sphingomyelin; phosphatidylinositol; phosphatidylcholine; sulfatide; cardiolipin) in order to show that GPR56 specifically binds PS. Also, the authors use FITC-conjugated Annexin V as a positive control but do not show its binding to Ba/F3 cells (Fig. 1D). Finally, the use of primary neurons instead than Ba/F3 could be more relevant in the present context.*

Response: We appreciate the suggestions. Unfortunately, it is not possible to perform FACS analysis for other phospholipids due to the lack of fluorophore-tagged reagents. Instead, we performed a protein-lipid overlay experiment using Membrane Lipid Strips and included the data in this revised manuscript (Please see Page 8, line 3-14).

Given that FITC-conjugated Annexin V was in different channel as AF647 and cannot be combined in Fig. C. We have since include the data in Appendix Fig.S2.

Indeed, primary neurons would be more relevant. However, we do not have the ability to reliably induce primary neurons undergo PS externalization. Furthermore, our goal is to demonstrate GPR56 can binds phosphatidylserine on live cells. In this regard, Ba/F3 cell line is well established cell-based model system (Gyobu et al., 2017; Miyanishi et al., 2007; Suzuki et al., 2010).

5. *Fig. 3: a functional characterization of microglia lacking GPR56 or GPR56 S4 would be essential. Is the general phagocytic activity of microglia impacted by the lack of either protein? Is the inflammatory profile changed?*

Response: Our *in vivo* engulfment assay showed that microglial GPR56 conditional ko (CKO) mice exhibited impaired phagocytosis of RGC inputs (See Fig. EV 5C and D). Interestingly, microglia lacking GPR56 didn't show significant defects in general cellular properties, like cellular density, %CD68, morphology (See Fig. EV 3C-J).

6. *Fig. 3I: The authors analyzed GPR56 S4 transcript in microglia at P14 in WT mice. Which brain area is microglia isolated from? Does GPR56 expression vary at P5, when microglia are actively pruning synapse in the dLGN, with respect to P13?*

Response: We thank the reviewer for the comments. We included experimental details in the method section in this revised manuscript. "Microglia were isolated from whole brains without cerebellum" (Page 25, line 11). Furthermore, we performed qPCR using P5 microglia, and showed that a steady increased of the transcript level from P5 to P14, which is consistent with previous reports (Fig. 3I, Appendix Fig. S1 and Bennet et al., PNAS 2016).

7. *Fig. 3 J-K: here the Authors, correctly, introduce the use of synaptic markers. However, defining the colocalizing Vglut1/homer1 structures as "synapse number" is not proper. The Authors should stick to the term "colocalizing Vglut1/homer1 puncta".*

Response: We agree with the reviewer's comment. In this revised manuscript, we specified colocalized vGlut2[+]/Homer1^{+} puncta as "retinogeniculate synapses" in the dLGN (Page 9, line 22), and vGlut1[+]/Homer1^{+} puncta as "corticogeniculate synapses" in the dLGN (Page 13, line 9).

8. *Fig. 3: The quantitation of synaptic puncta engulfed by microglia in GPR56 KO and S4 is mandatory, in order to state that "GPR56 S4 isoform is crucial to developmental synaptic refinement".*

Response: We thank the reviewer for the comment and have since performed the experiments and included our new data (Fig. 3M-O).

9. *Fig. 3: Also, although the Authors report that the increased synapse density is not the result of altered RGC number, given that GPR56 is involved in neuronal development, a quantitation of neuronal arborization should be performed in GPR56 KO and S4, to exclude that the differences in Vglut1/homer1 positive puncta depends on neuron-autonomous factors, different from synaptic pruning.*

Response: This request is reasonable in principle. Unfortunately, we found our inability to fulfill the request due to the following reasons:
1. Synapse number could alter without change in neuronal arborization. The synapses we analyzed are vGlut2$^+$ presynaptic inputs from RGCs. In early postnatal stage, the number of synapses decreases dramatically, but the average RGC arbor size and complexity remain largely unchanged (Hong et al., 2014). RGCs exhibit many *en passant* synapses (synapses on the stem of the RGC axons) that undergo elimination without changing the axonal structure (Hong et al., 2014).

2. Golgi staining will not work in this case, because it can't differentiate RGC arbors from dendritic arbors of local relay neurons and cortical neuronal arbors in the dLGN. Importantly, cortical inputs comprise ~90% synapses in dLGN.

3. It seems to us that generating RGC reporter line to visualize arbors as used in Hong et al., Eur J Neurosci 2019 would be an option. However, this will require up to 1-2 years of time to cross the reporter line into Gpr56 KO and S4 mutant background. Furthermore, there are over 20 different types of RGC cells, and different RGC classes exhibit distinct arborization patterns in the dLGN (Hong et al., 2019), and it is impossible for us to determine which type of RGC cells to study.

4. It is correct that GPR56 is expressed in first born neurons in the developing neocortex. However, it is largely absent in mature neurons. Furthermore, to address neuron-autonomous factors, we generated both microglial *Gpr56* constitutive conditional ko and microglial *Gpr56* tamoxifen-inducible ko mice, where neuronal *Gpr56* expression was not affected. We observed similar synapse defects as seen in *Gpr56* global ko mice, supporting that the synaptic chance was NOT due to "neuron-autonomous factors".

10. *Fig. 3: Also, based on the decrease in MBP in the corpus callosum, the possibility that demyelination might impact synaptic pruning by microglia should be at least discussed.*

Response: We thank the reviewer for the comment. We discussed it in the main text (please see Page 18, line 6-12).

11. *Fig. 3K and L: The images in Fig. 3K show an apparent higher density of Vglut2+ puncta in Gpr56 null mice, while Homer1+ puncta seem to be increased in both GPR56 null and GPR56 S4. A quantification of Vglut2 or Homer1 puncta, separately, could be of interest to investigate the possibility that GPR56 may be involved in mediating predominantly the elimination of presynaptic or postsynaptic puncta.*

Response: We quantified the densities of vGlut2$^+$ presynaptic inputs and homer1$^+$ postsynaptic signals, and only found increased vGlut2$^+$, but not homer1$^+$ in *Gpr56 null* (see new Appendix Figure S4, Page 10, line 1-2).

12. *Fig* S3 and Bennet et al., PNAS 2016, show that GPR56 transcript increases in microglia during development. The authors should discuss the meaning of this increase, since, according to their hypothesis, GPR56 is critical during the synaptic pruning period.

Response: We appreciate this comment. *Gpr56* transcript increases in microglia from embryonic stage and reaches a relatively high level between P3-P6, a period of active microglia-mediated synaptic pruning (Appendix Fig. S1, Page 7, line 1-2).

13. *Fig. 4: while this figure is consistent with the concept that "Deleting microglial GPR56 results in excess synapses in the dorsolateral geniculate nucleus during postnatal development", again no evidence is presented that the increased synaptic density is the consequence of a defect in synaptic pruning. Some experiments would be crucial to address this possibility: qPCR should be performed to demonstrate that the higher synaptic protein expression does not result from increased synaptic protein transcription in mutant mice. Even more importantly, analysis of synaptic engulfment by microglia should be performed by quantifying the synaptic markers internalized in microglial CD68-positive structures.*

Response: We appreciate this suggestion. However, we are constrained by technical limitations. Dorothy Schafer showed that most proteins engulfed by microglia will be quickly degraded once in lysosomes, which makes it unreliable to do synaptic engulfment analysis (Schafer et al., 2014). On the contrary, Alexa dye is more resistant to lysosomal hydrolases (Mukhopadhyay et al., 2010), making Alexa conjugated CTB as a robust dye to label engulfed material. Therefore, we performed our microglial engulfment assay using state of the art technology and showed a decreased engulfment in microglial GPR56 ko mice (Please see Fig. EV5C and D).

14. *Fig. 4 C and D: A quantification of Vglut2+ or Homer1+ puncta, separately, could be of interest (see above).*

Response: We included the quantification of Vglut2$^+$ or Homer1$^+$ puncta as a new Appendix Fig. S6.

15. *Fig. 3*: 4 E-H: Quantification by structured illumination microscopy (SIM) was performed in P8 mice brains, while WB analysis of CTR and CKO mice was performed at P30. The authors should add WB quantification of Vglut2 in the dLGN also at P8. Also, WB quantification of postsynaptic markers should be added, together with qPCR analyses for both pre and postsynaptic protein mRNAs.

Response: We performed the experiment as suggested and included the new data in this revised manuscript (Please see new Fig. 4 G-I, Page 12, line 6-9).

16. *Page 14: "Considering that PS binds GPR56 and flags synapses for removal by microglia" This sentence should be modified. The authors only showed that PSVue+ puncta colocalize with CTB-488. They did not perform staining with synaptic markers and omitted to do additional controls (see comments to Fig.1).*

Response: We appreciate the suggestion and modified the sentence as "Considering that PS binds GPR56 and flags RGC presynaptic inputs for removal by microglia" (Page 15, line 18).

17. *Fig. 5: this figure should be removed. A convincing demonstration that "microglial GPR56 regulates hippocampal synaptic development in a circuit-dependent manner" would require significantly deeper analyses (characterization of GPR56 expression in different hippocampal regions, quantitation of microglial engulfed synaptic material, Western Blotting, qPCR and so on).*

Response: In response to the reviewer's comment and editor's guidance, we have reworded our statement as "Microglial GPR56 regulates hippocampal synaptic development" (Page 13, line 14).

18. *Fig.* 7A-B: Please, see the comments above (Figure 1 C-D)

Response: As the same as Fig 1C-D, the measure was normalized to total number of RGC inputs.

19. *Fig. 8: the scheme provided is far ahead of the actual results reported in this manuscript.*

Response: We respectively disagree with this comment. Gratefully, we thank our editor Dr. Karin Dumstrei on her encouragement of including this figure.

20. *Page 6: Please provide the expanded definition for NTF. The figure legends 2 A and B should explain better how the authors engineered recombinant proteins.*

Response: We added the full name "N-terminal fragment" for NTF in the main text (Page 7, line 7). And in the Figure legend 2, we changed it to "(A) A schematic drawing of GPR56 protein structure, with a N-terminal fragment (NTF), a seven transmembrane domain (7-TM) and a C-terminal fragment (CTF). (B) A diagram shows the hFc tag was added to the c-terminal of GPR56-NTF (NTF-hFC) or GAIN domain (GAIN-hFc)." (Page 36, line 2-5)

21. *Fig. 4 A and B. Which area were analyzed? What is the mice's age? Please, specify this in the text and fig. legends.*

Response: We added the details of brain area and age in the main text: "We further performed RNAscope analysis for *Gpr56* in the prefrontal cortex of P30 mice" (Page 10, line 21-22). In the figure legends 4, we added "RNAscope was performed in the prefrontal cortex of P30 mice." (Page 39, line 5)

22. *Pag. 11: Please provide the expanded definition for Brn3a+*

Response: We added the definition for Bra3a+ in the main text. "brain-specific homeobox/POU domain protein 3A positive (Brn3a^{+}, a marker of RGC). (Page 12, line 10)

Reviewer #2:
1. *Using PSVue to label PS presynaptic elements or PS on BA/F3 cells, the authors show that the GAIN domain of GPR56 binds PS. For the in vivo synaptic pruning experiments, the authors use the widely used anterograde tracer cholera toxin B to show PS+ engulfment. Could the authors address if there are any confounders about CTB binding lipid rafts which presumably PSVue may also bind? For example, to show that PS+ terminals are indeed presynaptic phagocytosed elements, combining analysis of PS+ Homer+ elements within microglia?*

Response: We appreciated the reviewer for this thoughtful comment. To demonstrate the specificity of PSVue binding to synapses, we performed additional experiment, Iba1/CD68/vGlut2/PSVue co-staining, and showed PSVue[+]/vGlut2[+] retinogeniculate synapses colocalized with CD68, and phagocyted by microglia (Please see new Fig. 1H, Page 6, line 15). Considering the fact that CTB binds lipid rafts, we

would expect to observe a similar colocalization between P6 and P13 if PSVue also binds to lipid rafts. However, we observed that PSVue only colocalized with ~10% CTB at P6 and 3% at P13.

> 2. *It would be helpful to include a diagram as in Fig3A with the GPR56 NULL mouse for comparison.*

Response: We thank the reviewer for the thoughtful suggestion and included a diagram in Fig 3A in this revised manuscript.

> 3. *As the stated justification to use CX3CR1-Cre, this reviewer would recommend authors not necessarily use Zhao et al 2019 as proof that it is microglia specific, since the reasoning is less likely non-specific recombination of the reporter but rather different sensitivity of reporters; would instead recommend that authors emphasize their efforts to show microglia specific knockout.*

Response: We thank the reviewer for the suggestion. We removed the paper of Zhao et al 2019 and included the discussion on our generation of the microglial *Gpr56* specific knockout mice. (Page 10, paragraph 3).

> 4. *Using the RNAscope to show loss of GPR56 in microglia… is not, however, showing microglia-specificity, as an analysis of # non-microglial cells expressing GPR56 would be a more valid approach. Unless looking at recombination, however, would rephrase explanation that their approach unlikely non-specifically targeting cells in the retinogeniculate system given that GPR56 levels in non-microglial cells are comparable.*

Response: We appreciate reviewer's suggestion. In this revised manuscript, we included a new Fig. 4B and rephrased our discussion (Page 10, line 21-23; Page 11, line 1-2).

> 5. *Is the % increase in synaptic number observed for CKO for the various methods (Homer/VGlut1, SIM) comparable?*

Response: The % of increase was different but comparable between SIM and confocal data. Specifically, we observed ~35% increase in CKO at P10 using traditional confocal imaging, and a ~50% increase in CKO at P8 using SIM. The small difference could result from age differences (P10 vs P8), and/or from method differences (traditional confocal vs SIM).

> 6. *In the abstract, the authors state that developmental synaptic remodeling defects lead to neurodevelopmental disorders. This struck me as a bit absolute - not all neurodevelopmental are caused by synaptic pruning defects, and it is largely an implication that this process underlies neurodevelopmental disorders.. Rather than direct lines of proof. The authors write with a more balanced voice in the Concluding remarks and I would recommend they slightly edit the language in the abstract to respect this nuance.*

Response: We appreciate the comments and have since reworded "Developmental synaptic remodeling is important for the formation of precise neural circuitry and its disruption has been linked to neurodevelopmental disorders such as autism and schizophrenia." (Page 2, line 1-3).

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Dear Xian,

Thanks for sending me the revised manuscript. I have now had a chance to take a look at it and I appreciate the introduced changes. I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
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A splicing isoform of GPR56 mediates microglial synaptic refinement via phosphatidylserine binding

Tao Li, Brian Chiou, Casey Gilman, Rong Luo, Diankun Yu, Stefanie Giera, Erin Johnson-Venkatesh, Allie Muthukumar, Beth Stevens, Hisashi Umemori, Xianhua Piao, Tatsuhiro Koshi, and Hayeon Oak
DOI: N/A

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I am happy to discuss the revisions further.

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Thank you for the opportunity to consider your work for publication. I look forward to your revision.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

In the present manuscript, Tao Li and Colleagues describe a novel role for the adhesion G protein-coupled receptor (aGPCR) ADGRG1/GPR56 expressed on microglial cells. By in vitro binding experiments and in vivo GPR56 full knockout or conditional knockout mice, they show that the alternatively spliced isoform of GPR56 (GPR56 S4) is required for microglia-mediated synapse elimination during postnatal life in the mouse. They also report that GPR56 S4 binds phosphatidylserine (PS) and that PS serves as an "eat-me" signal for synapse engulfment by microglia. Although the identification of PS as putative signal for synapse elimination and the clarification of the role of microglial GPR56 in the process would represent a major advancement in the field, the study lacks crucial experiments to support the drawn conclusion.

Figure 1 and page 5: The authors claim that PS is a new "eat-me" signal exposed at the synapse in the developing brain, based on the use of the PS marker PSVue and the anterograde tracer fluorescent cholera toxin B (CTB). Although the Authors show that some RGC inputs in the dLGN of WT mice are labeled by PSVue and that the extent of labeling reduces from P6 to P13 (thus paralleling the timing of microglia-mediated synapse engulfment) no evidence for synaptic labeling by PSVue is provided. The authors should stain brain sections with antibodies against either pre or

postsynaptic markers and show their colocalization with PSVue.

Karin: This issue is raised by both referees #1 and 2 - please address.

Fig. 1 C-D: It is known that in the dLNG microglial engulfment of synaptic elements peaks at P5 and is decreased at P10 (Schafer et al., 2012) and in fact the density of synaptic contacts is reduced in parallel. If CTB-488 positive structures represent synaptic terminals, a reduction of CTB-488 staining would be expected in P13 dLGN. The graph shows the % PS+ RGC inputs. Has this measure been normalized to the total number of CTB-488+ inputs?

Karin: please clarify

Fig. 1 E-F: synaptic engulfment should be demonstrated by showing synaptic markers internalized in microglial CD68-positive structures. Orthogonal sections should be shown and analyzed. The authors observe ~73% PS+ and 27% PS- RGC inputs within microglia. A parallel quantification of PVSue+ and RGC+ inputs inside and outside microglia is needed. Finally, 5-TAMRA does not prove that PSVue is specifically exposed at the synapse.

Karin: This point would be good to address and it looks like you have the tools on hand to address this issue. Let me know if there are any problems with doing the experiments

Fig. 2: this experiment lacks negative controls. The authors should perform the same experiments using different phospholipids (i.e. sphingomyelin; phosphatidylinositol; phosphatidylcholine; sulfatide; cardiolipin) in order to show that GPR56 specifically binds PS. Also, the authors use FITC-conjugated Annexin V as a positive control but do not show its binding to Ba/F3 cells (Fig. 1D). Finally, the use of primary neurons instead than Ba/F3 could be more relevant in the present context.

Karin: fine to use Ba/F3 cells no need to extend to primary cells. Would be good to test different phospholipids

Fig. 3: a functional characterization of microglia lacking GPR56 or GPR56 S4 would be essential. Is the general phagocytic activity of microglia impacted by the lack of either protein? Is the inflammatory profile changed?

Karin: if you have data on hand to address this point then please include it. If not then OK not to address this issue.

Fig. 3I: The authors analyzed GPR56 S4 transcript in microglia at P14 in WT mice. Which brain area is microglia isolated from? Does GPR56 expression vary at P5, when microglia are actively pruning synapse in the dLGN, with respect to P13?

Fig. 3 J-K: here the Authors, correctly, introduce the use of synaptic markers. However, defining the colocalizing Vglut1/homer1 structures as "synapse number" is not proper. The Authors should stick to the term "colocalizing Vglut1/homer1 puncta".

Karin: Please address

The quantitation of synaptic puncta engulfed by microglia in GPR56 KO and S4 is mandatory, in order to state that "GPR56 S4 isoform is crucial to developmental synaptic refinement". Also,

although the Authors report that the increased synapse density is not the result of altered RGC number, given that GPR56 is involved in neuronal development, a quantitation of neuronal arborization should be performed in GPR56 KO and S4, to exclude that the differences in Vglut1/homer1 positive puncta depends on neuron-autonomous factors, different from synaptic pruning. Also, based on the decrease in MBP in the corpus callosum, the possibility that demyelination might impact synaptic pruning by microglia should be at least discussed.

Karin: Please add quantification

Fig. 3K and L: The images in Fig. 3K show an apparent higher density of Vglut2+ puncta in Gpr56 null mice, while Homer1+ puncta seem to be increased in both GPR56 null and GPR56 S4. A quantification of Vglut2 or Homer1 puncta, separately, could be of interest to investigate the possibility that GPR56 may be involved in mediating predominantly the elimination of presynaptic or postsynaptic puncta.

Karin: If straight forward enough to do then please address this issue

Fig. S3 and Bennet et al., PNAS 2016, show that GPR56 transcript increases in microglia during development. The authors should discuss the meaning of this increase, since, according to their hypothesis, GPR56 is critical during the synaptic pruning period.

Karin: Please discuss

Fig. 4: while this figure is consistent with the concept that "Deleting microglial GPR56 results in excess synapses in the dorsolateral geniculate nucleus during postnatal development", again no evidence is presented that the increased synaptic density is the consequence of a defect in synaptic pruning. Some experiments would be crucial to address this possibility: qPCR should be performed to demonstrate that the higher synaptic protein expression does not result from increased synaptic protein transcription in mutant mice. Even more importantly, analysis of synaptic engulfment by microglia should be performed by quantifying the synaptic markers internalized in microglial CD68-positive structures.

Karin: I realise that this experiment is not straight forward but would be good to address

Fig. 4 C and D: A quantification of Vglut2+ or Homer1+ puncta, separately, could be of interest (see above).

Karin: see my comments to related point above

Fig. 4 E-H: Quantification by structured illumination microscopy (SIM) was performed in P8 mice brains, while WB analysis of CTR and CKO mice was performed at P30. The authors should add WB quantification of Vglut2 in the dLGN also at P8. Also, WB quantification of postsynaptic markers should be added, together with qPCR analyses for both pre and postsynaptic protein mRNAs.

Karin: please address

Page 14: "Considering that PS binds GPR56 and flags synapses for removal by microglia" This sentence should be modified. The authors only showed that PSVue+ puncta colocalize with CTB-488. They did not perform staining with synaptic markers and omitted to do additional controls (see comments to Fig.1).

Karin: please address

Fig. 5: this figure should be removed. A convincing demonstration that "microglial GPR56 regulates hippocampal synaptic development in a circuit-dependent manner" would require significantly deeper analyses (characterization of GPR56 expression in different hippocampal regions, quantitation of microglial engulfed synaptic material, Western Blotting, qPCR and so on).

Karin: I would suggest to leave the figure in as I do think it adds insight. make sure that the conclusions from this figure are not overstated

Fig. 7A-B: Please, see the comments above (Figure 1 C-D)

Fig. 8: the scheme provided is far ahead of the actual results reported in this manuscript.

Karin: I am OK with the figure and find it helpful. I would leave in.

Minor:

Page 6: Please provide the expanded definition for NTF. The figure legends 2 A and B should explain better how the authors engineered recombinant proteins.

Fig. 4 A and B. Which area were analyzed? What is the mice's age? Please, specify this in the text and fig. legends.

Minor: Pag. 11: Please provide the expanded definition for Brn3a+

Referee #2:

In this well-written and elegant study, Li and colleagues seek out to determine the function of microglial GPR56 in normal development. Having previously demonstrated the functions of GPR56 in cortical development and oligodendrocyte maturation, the authors ask whether microglial GPR56 is involved in normal synaptic pruning. They determined that a specific isoform of GPR56 (S4), containing only a GAIN domain, acts as a receptor for phosphatidylserine (PS+) which is required for most microglia-mediated phagocytosis of PS+ RGC synapses during retinogeniculate refinement. This isoform is preferentially expressed by microglia. The authors also link the defect in synapse pruning to functional consequences, and show that GPR56-mediated synaptic pruning is also at play in the developing hippocampus. Overall, this is a topic of wide interest to the readership of this journal, given growing interest in the function of microglia in development, health, and disease, and that this manuscript beautifully demonstrates the importance of splice variants in cell type specific functions of a G-protein coupled receptor expressed by multiple cell types. This reviewer has only a few concerns that if address would improve the impact of the study:
Using PSVue to label PS presynaptic elements or PS on BA/F3 cells, the authors show that the GAIN domain of GPR56 binds PS. For the invivo synaptic pruning experiments, the authors use the widely used anterograde tracer cholera toxin B to show PS+ engulfment. Could the authors address if there are any confounders about CTB binding lipid rafts which presumably PSVue may also bind?

For example, to show that PS+ terminals are indeed presynaptic phagocytosed elements, combining analysis of PS+ Homer+ elements within microglia?

It would be helpful to include a diagram as in Fig3A with the GPR56 NULL mouse for comparison.

As the stated justification to use CX3CR1-Cre, this reviewer would recommend authors not necessarily use Zhao et al 2019 as proof that it is microglia specific, since the reasoning is less likely non-specific recombination of the reporter but rather different sensitivity of reporters; would instead recommend that authors emphasize their efforts to show microglia specific knockout. Speaking of: Great work on using the RNAscope to show loss of GPR56 in microglia. This is not, however, showing microglia-specificity, as an analysis of # non-microglial cells expressing GPR56 would be a more valid approach. Unless looking at recombination, however, would rephrase explanation that their approach unlikely non-specifically targeting cells in the retinogeniculate system given that GPR56 levels in non-microglial cells are comparable.

Is the % increase in synaptic number observed for CKO for the various methods (Homer/VGlut1, SIM) comparable?

In the abstract, the authors state that developmental synaptic remodeling defects lead to neurodevelopmental disorders. This struck me as a bit absolute - not all neurodevelopmental are caused by synaptic pruning defects, and it is largely an implication that this process underlies neurodevelopmental disorders.. Rather than direct lines of proof. The authors write with a more balanced voice in the Concluding remarks and I would recommend they slightly edit the language in the abstract to respect this nuance.

We are grateful for the constructive comments from the reviewers. In response to the reviewers' comments, we have performed additional experiments. Below, please find our point-by-point responses to the reviewers' comments. In addition, we have revised the manuscript in accordance with these suggestions.

Reviewer #1

1. *Figure 1 and page 5: The authors claim that PS is a new "eat-me" signal exposed at the synapse in the developing brain, based on the use of the PS marker PSVue and the anterograde tracer fluorescent cholera toxin B (CTB). Although the Authors show that some RGC inputs in the dLGN of WT mice are labeled by PSVue and that the extent of labeling reduces from P6 to P13 (thus paralleling the timing of microglia-mediated synapse engulfment) no evidence for synaptic labeling by PSVue is provided. The authors should stain brain sections with antibodies against either pre or postsynaptic markers and show their colocalization with PSVue.*

Response: We appreciate the suggestion. We have performed the immunostaining of vGlut2 and Homer1 in P6 dLGN, and demonstrated that vGlut2 and Homer1 are colocalized with PSVue (Please see new Fig. 1E and F, Page 5, line 19-23).

2. *Fig. 1 C-D: It is known that in the dLGN microglial engulfment of synaptic elements peaks at P5 and is decreased at P10 (Schafer et al., 2012) and in fact the density of synaptic contacts is reduced in parallel. If CTB-488 positive structures represent synaptic terminals, a reduction of CTB-488 staining would be expected in P13 dLGN. The graph shows the % PS+ RGC inputs. Has this measure been normalized to the total number of CTB-488+ inputs?*

Response: The % PS$^+$ RGC inputs was normalized and calculated as the number of PS$^+$ RGC inputs in one field over the number of total RGC inputs in the same field. (Please see Figure legend 1(D), Page 35, line 9).

3. *Fig. 1 E-F: synaptic engulfment should be demonstrated by showing synaptic markers internalized in microglial CD68-positive structures. Orthogonal sections should be shown and analyzed. The authors observe ~73% PS+ and 27% PS- RGC inputs within microglia. A parallel quantification of PVSue+ and RGC+ inputs inside and outside microglia is needed. Finally, 5-TAMRA does not prove that PSVue is specifically exposed at the synapse.*

Response: We are thankful for the suggestions. We have replaced the word "synapse" with "RGC synaptic inputs" in the text (Please see Page 4-6). We performed Iba1/CD68/CTB/PSVue co-staining, and showed PSVue$^+$ RGC inputs colocalized with CD68 and Iba1 (Please see new Fig. 1G, and page 6, line 13-15). We also carried out Iba1/CD68/vGlut2/PSVue co-staining, and showed PSVue$^+$/vGlut2$^+$ retinogeniculate synapses colocalized with CD68 and Iba1 (Please see new Fig. 1H, and Page 6, line 15). We also performed parallel quantification of PSVue$^+$ RGC inputs inside as well as outside of microglia (Fig. 1I and J, Page 6. line 16-19).

With regards to "*5-TAMRA does not prove that PSVue is specifically exposed at the synapse,*" we apologize for the confusion. We have revised and reemphasized that we used 5-TAMRA as a negative control and found that very little 5-TAMRA signal colocalized with RGC inputs and engulfed by microglia, which suggested that microglia don't engulf free fluorophore and the PSVue signals inside microglia were not free fluorophore (Page 6, line 6-11).

4. *Fig. 2: this experiment lacks negative controls. The authors should perform the same experiments using different phospholipids (i.e. sphingomyelin; phosphatidylinositol; phosphatidylcholine; sulfatide; cardiolipin) in order to show that GPR56 specifically binds PS. Also, the authors use FITC-conjugated Annexin V as a positive control but do not show its binding to Ba/F3 cells (Fig. 1D). Finally, the use of primary neurons instead than Ba/F3 could be more relevant in the present context.*

Response: We appreciate the suggestions. Unfortunately, it is not possible to perform FACS analysis for other phospholipids due to the lack of fluorophore-tagged reagents. Instead, we performed a protein-lipid overlay experiment using Membrane Lipid Strips and included the data in this revised manuscript (Please see Page 8, line 3-14).

Given that FITC-conjugated Annexin V was in different channel as AF647 and cannot be combined in Fig. C. We have since include the data in Appendix Fig.S2.

Indeed, primary neurons would be more relevant. However, we do not have the ability to reliably induce primary neurons undergo PS externalization. Furthermore, our goal is to demonstrate GPR56 can binds phosphatidylserine on live cells. In this regard, Ba/F3 cell line is well established cell-based model system (Gyobu et al., 2017; Miyanishi et al., 2007; Suzuki et al., 2010).

5. *Fig. 3: a functional characterization of microglia lacking GPR56 or GPR56 S4 would be essential. Is the general phagocytic activity of microglia impacted by the lack of either protein? Is the inflammatory profile changed?*

Response: Our *in vivo* engulfment assay showed that microglial GPR56 conditional ko (CKO) mice exhibited impaired phagocytosis of RGC inputs (See Fig. EV 5C and D). Interestingly, microglia lacking GPR56 didn't show significant defects in general cellular properties, like cellular density, %CD68, morphology (See Fig. EV 3C-J).

6. *Fig. 3I: The authors analyzed GPR56 S4 transcript in microglia at P14 in WT mice. Which brain area is microglia isolated from? Does GPR56 expression vary at P5, when microglia are actively pruning synapse in the dLGN, with respect to P13?*

Response: We thank the reviewer for the comments. We included experimental details in the method section in this revised manuscript. "Microglia were isolated from whole brains without cerebellum" (Page 25, line 11). Furthermore, we performed qPCR using P5 microglia, and showed that a steady increased of the transcript level from P5 to P14, which is consistent with previous reports (Fig. 3I, Appendix Fig. S1 and Bennet et al., PNAS 2016).

7. *Fig. 3 J-K: here the Authors, correctly, introduce the use of synaptic markers. However, defining the colocalizing Vglut1/homer1 structures as "synapse number" is not proper. The Authors should stick to the term "colocalizing Vglut1/homer1 puncta".*

Response: We agree with the reviewer's comment. In this revised manuscript, we specified colocalized vGlut2[+]/Homer1^{+} puncta as "retinogeniculate synapses" in the dLGN (Page 9, line 22), and vGlut1[+]/Homer1^{+} puncta as "corticogeniculate synapses" in the dLGN (Page 13, line 9).

8. *Fig. 3: The quantitation of synaptic puncta engulfed by microglia in GPR56 KO and S4 is mandatory, in order to state that "GPR56 S4 isoform is crucial to developmental synaptic refinement".*

Response: We thank the reviewer for the comment and have since performed the experiments and included our new data (Fig. 3M-O).

9. *Fig. 3: Also, although the Authors report that the increased synapse density is not the result of altered RGC number, given that GPR56 is involved in neuronal development, a quantitation of neuronal arborization should be performed in GPR56 KO and S4, to exclude that the differences in Vglut1/homer1 positive puncta depends on neuron-autonomous factors, different from synaptic pruning.*

Response: This request is reasonable in principle. Unfortunately, we found our inability to fulfill the request due to the following reasons:
1. Synapse number could alter without change in neuronal arborization. The synapses we analyzed are vGlut2^{+} presynaptic inputs from RGCs. In early postnatal stage, the number of synapses decreases dramatically, but the average RGC arbor size and complexity remain largely unchanged (Hong et al., 2014). RGCs exhibit many *en passant* synapses (synapses on the stem of the RGC axons) that undergo elimination without changing the axonal structure (Hong et al., 2014).

2. Golgi staining will not work in this case, because it can't differentiate RGC arbors from dendritic arbors of local relay neurons and cortical neuronal arbors in the dLGN. Importantly, cortical inputs comprise ~90% synapses in dLGN.

3. It seems to us that generating RGC reporter line to visualize arbors as used in Hong et al., Eur J Neurosci 2019 would be an option. However, this will require up to 1-2 years of time to cross the reporter line into Gpr56 KO and S4 mutant background. Furthermore, there are over 20 different types of RGC cells, and different RGC classes exhibit distinct arborization patterns in the dLGN (Hong et al., 2019), and it is impossible for us to determine which type of RGC cells to study.

4. It is correct that GPR56 is expressed in first born neurons in the developing neocortex. However, it is largely absent in mature neurons. Furthermore, to address neuron-autonomous factors, we generated both microglial *Gpr56* constitutive conditional ko and microglial *Gpr56* tamoxifen-inducible ko mice, where neuronal *Gpr56* expression was not affected. We observed similar synapse defects as seen in *Gpr56* global ko mice, supporting that the synaptic chance was NOT due to "neuron-autonomous factors".

10. *Fig. 3: Also, based on the decrease in MBP in the corpus callosum, the possibility that demyelination might impact synaptic pruning by microglia should be at least discussed.*

Response: We thank the reviewer for the comment. We discussed it in the main text (please see Page 18, line 6-12).

11. *Fig. 3K and L: The images in Fig. 3K show an apparent higher density of Vglut2+ puncta in Gpr56 null mice, while Homer1+ puncta seem to be increased in both GPR56 null and GPR56 S4. A quantification of Vglut2 or Homer1 puncta, separately, could be of interest to investigate the possibility that GPR56 may be involved in mediating predominantly the elimination of presynaptic or postsynaptic puncta.*

Response: We quantified the densities of vGlut2$^+$ presynaptic inputs and homer1$^+$ postsynaptic signals, and only found increased vGlut2$^+$, but not homer1$^+$ in *Gpr56 null* (see new Appendix Figure S4, Page 10, line 1-2).

12. *Fig* S3 and Bennet et al., PNAS 2016, show that GPR56 transcript increases in microglia during development. The authors should discuss the meaning of this increase, since, according to their hypothesis, GPR56 is critical during the synaptic pruning period.

Response: We appreciate this comment. *Gpr56* transcript increases in microglia from embryonic stage and reaches a relatively high level between P3-P6, a period of active microglia-mediated synaptic pruning (Appendix Fig. S1, Page 7, line 1-2).

13. *Fig. 4: while this figure is consistent with the concept that "Deleting microglial GPR56 results in excess synapses in the dorsolateral geniculate nucleus during postnatal development", again no evidence is presented that the increased synaptic density is the consequence of a defect in synaptic pruning. Some experiments would be crucial to address this possibility: qPCR should be performed to demonstrate that the higher synaptic protein expression does not result from increased synaptic protein transcription in mutant mice. Even more importantly, analysis of synaptic engulfment by microglia should be performed by quantifying the synaptic markers internalized in microglial CD68-positive structures.*

Response: We appreciate this suggestion. However, we are constrained by technical limitations. Dorothy Schafer showed that most proteins engulfed by microglia will be quickly degraded once in lysosomes, which makes it unreliable to do synaptic engulfment analysis (Schafer et al., 2014). On the contrary, Alexa dye is more resistant to lysosomal hydrolases (Mukhopadhyay et al., 2010), making Alexa conjugated CTB as a robust dye to label engulfed material. Therefore, we performed our microglial engulfment assay using state of the art technology and showed a decreased engulfment in microglial GPR56 ko mice (Please see Fig. EV5C and D).

14. *Fig. 4 C and D: A quantification of Vglut2+ or Homer1+ puncta, separately, could be of interest (see above).*

Response: We included the quantification of Vglut2$^+$ or Homer1$^+$ puncta as a new Appendix Fig. S6.

15. *Fig. 3*: 4 E-H: Quantification by structured illumination microscopy (SIM) was performed in P8 mice brains, while WB analysis of CTR and CKO mice was performed at P30. The authors should add WB quantification of Vglut2 in the dLGN also at P8. Also, WB quantification of postsynaptic markers should be added, together with qPCR analyses for both pre and postsynaptic protein mRNAs.

Response: We performed the experiment as suggested and included the new data in this revised manuscript (Please see new Fig. 4 G-I, Page 12, line 6-9).

16. *Page 14: "Considering that PS binds GPR56 and flags synapses for removal by microglia" This sentence should be modified. The authors only showed that PSVue+ puncta colocalize with CTB-488. They did not perform staining with synaptic markers and omitted to do additional controls (see comments to Fig.1).*

Response: We appreciate the suggestion and modified the sentence as "Considering that PS binds GPR56 and flags RGC presynaptic inputs for removal by microglia" (Page 15, line 18).

17. *Fig. 5: this figure should be removed. A convincing demonstration that "microglial GPR56 regulates hippocampal synaptic development in a circuit-dependent manner" would require significantly deeper analyses (characterization of GPR56 expression in different hippocampal regions, quantitation of microglial engulfed synaptic material, Western Blotting, qPCR and so on).*

Response: In response to the reviewer's comment and editor's guidance, we have reworded our statement as "Microglial GPR56 regulates hippocampal synaptic development" (Page 13, line 14).

18. *Fig.* 7A-B: Please, see the comments above (Figure 1 C-D)

Response: As the same as Fig 1C-D, the measure was normalized to total number of RGC inputs.

19. *Fig. 8: the scheme provided is far ahead of the actual results reported in this manuscript.*

Response: We respectively disagree with this comment. Gratefully, we thank our editor Dr. Karin Dumstrei on her encouragement of including this figure.

20. *Page 6: Please provide the expanded definition for NTF. The figure legends 2 A and B should explain better how the authors engineered recombinant proteins.*

Response: We added the full name "N-terminal fragment" for NTF in the main text (Page 7, line 7). And in the Figure legend 2, we changed it to "(A) A schematic drawing of GPR56 protein structure, with a N-terminal fragment (NTF), a seven transmembrane domain (7-TM) and a C-terminal fragment (CTF). (B) A diagram shows the hFc tag was added to the c-terminal of GPR56-NTF (NTF-hFC) or GAIN domain (GAIN-hFc)." (Page 36, line 2-5)

21. *Fig. 4 A and B. Which area were analyzed? What is the mice's age? Please, specify this in the text and fig. legends.*

Response: We added the details of brain area and age in the main text: "We further performed RNAscope analysis for *Gpr56* in the prefrontal cortex of P30 mice" (Page 10, line 21-22). In the figure legends 4, we added "RNAscope was performed in the prefrontal cortex of P30 mice." (Page 39, line 5)

22. *Pag. 11: Please provide the expanded definition for Brn3a+*

Response: We added the definition for Bra3a+ in the main text. "brain-specific homeobox/POU domain protein 3A positive (Brn3a$^+$, a marker of RGC). (Page 12, line 10)

Reviewer #2:
1. *Using PSVue to label PS presynaptic elements or PS on BA/F3 cells, the authors show that the GAIN domain of GPR56 binds PS. For the in vivo synaptic pruning experiments, the authors use the widely used anterograde tracer cholera toxin B to show PS+ engulfment. Could the authors address if there are any confounders about CTB binding lipid rafts which presumably PSVue may also bind? For example, to show that PS+ terminals are indeed presynaptic phagocytosed elements, combining analysis of PS+ Homer+ elements within microglia?*

Response: We appreciated the reviewer for this thoughtful comment. To demonstrate the specificity of PSVue binding to synapses, we performed additional experiment, Iba1/CD68/vGlut2/PSVue co-staining, and showed PSVue$^+$/vGlut2$^+$ retinogeniculate synapses colocalized with CD68, and phagocyted by microglia (Please see new Fig. 1H, Page 6, line 15). Considering the fact that CTB binds lipid rafts, we

would expect to observe a similar colocalization between P6 and P13 if PSVue also binds to lipid rafts. However, we observed that PSVue only colocalized with ~10% CTB at P6 and 3% at P13.

> 2. *It would be helpful to include a diagram as in Fig3A with the GPR56 NULL mouse for comparison.*

Response: We thank the reviewer for the thoughtful suggestion and included a diagram in Fig 3A in this revised manuscript.

> 3. *As the stated justification to use CX3CR1-Cre, this reviewer would recommend authors not necessarily use Zhao et al 2019 as proof that it is microglia specific, since the reasoning is less likely non-specific recombination of the reporter but rather different sensitivity of reporters; would instead recommend that authors emphasize their efforts to show microglia specific knockout.*

Response: We thank the reviewer for the suggestion. We removed the paper of Zhao et al 2019 and included the discussion on our generation of the microglial *Gpr56* specific knockout mice. (Page 10, paragraph 3).

> 4. *Using the RNAscope to show loss of GPR56 in microglia… is not, however, showing microglia-specificity, as an analysis of # non-microglial cells expressing GPR56 would be a more valid approach. Unless looking at recombination, however, would rephrase explanation that their approach unlikely non-specifically targeting cells in the retinogeniculate system given that GPR56 levels in non-microglial cells are comparable.*

Response: We appreciate reviewer's suggestion. In this revised manuscript, we included a new Fig. 4B and rephrased our discussion (Page 10, line 21-23; Page 11, line 1-2).

> 5. *Is the % increase in synaptic number observed for CKO for the various methods (Homer/VGlut1, SIM) comparable?*

Response: The % of increase was different but comparable between SIM and confocal data. Specifically, we observed ~35% increase in CKO at P10 using traditional confocal imaging, and a ~50% increase in CKO at P8 using SIM. The small difference could result from age differences (P10 vs P8), and/or from method differences (traditional confocal vs SIM).

> 6. *In the abstract, the authors state that developmental synaptic remodeling defects lead to neurodevelopmental disorders. This struck me as a bit absolute - not all neurodevelopmental are caused by synaptic pruning defects, and it is largely an implication that this process underlies neurodevelopmental disorders.. Rather than direct lines of proof. The authors write with a more balanced voice in the Concluding remarks and I would recommend they slightly edit the language in the abstract to respect this nuance.*

Response: We appreciate the comments and have since reworded "Developmental synaptic remodeling is important for the formation of precise neural circuitry and its disruption has been linked to neurodevelopmental disorders such as autism and schizophrenia." (Page 2, line 1-3).

Reference

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Suzuki, J., Umeda, M., Sims, P.J., and Nagata, S. (2010). Calcium-dependent phospholipid scrambling by TMEM16F. Nature 468, 834-838.

Dear Xianhua,

Thanks for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referee #2 and as you can see from the comments below, the the referee appreciate the introduced changes and supports publication here. I am therefore pleased to accept the manuscript for publication here. Before sending you the formal accept letter there are just a few editorial comments to take care of.

- Keywords are missing

- Author contribution is missing for Tatsuhiro Koshi

- For the data availability section: Please state "This study includes no data deposited in external repositories"

- The appendix figure files need to be added together in one appendix that also includes the legends from the figures. This file should also have a ToC

- The movie file needs to be zipped together with its legend.

- We also include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by 400 high (pixels). You can also use something from the figures if that is easier.

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That should be all. Let me know if you have any further questions.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
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Referee #2:

As this is a re-review, I will keep it brief:

The authors have sufficiently addressed this reviewer's concerns in their revised manuscript.

We are grateful for the constructive comments from the reviewers. In response to the reviewers' comments, we have performed additional experiments. Below, please find our point-by-point responses to the reviewers' comments. In addition, we have revised the manuscript in accordance with these suggestions.

Reviewer #1

1. *Figure 1 and page 5: The authors claim that PS is a new "eat-me" signal exposed at the synapse in the developing brain, based on the use of the PS marker PSVue and the anterograde tracer fluorescent cholera toxin B (CTB). Although the Authors show that some RGC inputs in the dLGN of WT mice are labeled by PSVue and that the extent of labeling reduces from P6 to P13 (thus paralleling the timing of microglia-mediated synapse engulfment) no evidence for synaptic labeling by PSVue is provided. The authors should stain brain sections with antibodies against either pre or postsynaptic markers and show their colocalization with PSVue.*

Response: We appreciate the suggestion. We have performed the immunostaining of vGlut2 and Homer1 in P6 dLGN, and demonstrated that vGlut2 and Homer1 are colocalized with PSVue (Please see new Fig. 1E and F, Page 5, line 19-23).

2. *Fig. 1 C-D: It is known that in the dLGN microglial engulfment of synaptic elements peaks at P5 and is decreased at P10 (Schafer et al., 2012) and in fact the density of synaptic contacts is reduced in parallel. If CTB-488 positive structures represent synaptic terminals, a reduction of CTB-488 staining would be expected in P13 dLGN. The graph shows the % PS+ RGC inputs. Has this measure been normalized to the total number of CTB-488+ inputs?*

Response: The % PS$^+$ RGC inputs was normalized and calculated as the number of PS$^+$ RGC inputs in one field over the number of total RGC inputs in the same field. (Please see Figure legend 1(D), Page 35, line 9).

3. *Fig. 1 E-F: synaptic engulfment should be demonstrated by showing synaptic markers internalized in microglial CD68-positive structures. Orthogonal sections should be shown and analyzed. The authors observe ~73% PS+ and 27% PS- RGC inputs within microglia. A parallel quantification of PVSue+ and RGC+ inputs inside and outside microglia is needed. Finally, 5-TAMRA does not prove that PSVue is specifically exposed at the synapse.*

Response: We are thankful for the suggestions. We have replaced the word "synapse" with "RGC synaptic inputs" in the text (Please see Page 4-6). We performed Iba1/CD68/CTB/PSVue co-staining, and showed PSVue$^+$ RGC inputs colocalized with CD68 and Iba1 (Please see new Fig. 1G, and page 6, line 13-15). We also carried out Iba1/CD68/vGlut2/PSVue co-staining, and showed PSVue$^+$/vGlut2$^+$ retinogeniculate synapses colocalized with CD68 and Iba1 (Please see new Fig. 1H, and Page 6, line 15). We also performed parallel quantification of PSVue$^+$ RGC inputs inside as well as outside of microglia (Fig. 1I and J, Page 6. line 16-19).

With regards to "*5-TAMRA does not prove that PSVue is specifically exposed at the synapse,*" we apologize for the confusion. We have revised and reemphasized that we used 5-TAMRA as a negative control and found that very little 5-TAMRA signal colocalized with RGC inputs and engulfed by microglia, which suggested that microglia don't engulf free fluorophore and the PSVue signals inside microglia were not free fluorophore (Page 6, line 6-11).

4. *Fig. 2: this experiment lacks negative controls. The authors should perform the same experiments using different phospholipids (i.e. sphingomyelin; phosphatidylinositol; phosphatidylcholine; sulfatide; cardiolipin) in order to show that GPR56 specifically binds PS. Also, the authors use FITC-conjugated Annexin V as a positive control but do not show its binding to Ba/F3 cells (Fig. 1D). Finally, the use of primary neurons instead than Ba/F3 could be more relevant in the present context.*

Response: We appreciate the suggestions. Unfortunately, it is not possible to perform FACS analysis for other phospholipids due to the lack of fluorophore-tagged reagents. Instead, we performed a protein-lipid overlay experiment using Membrane Lipid Strips and included the data in this revised manuscript (Please see Page 8, line 3-14).

Given that FITC-conjugated Annexin V was in different channel as AF647 and cannot be combined in Fig. C. We have since include the data in Appendix Fig.S2.

Indeed, primary neurons would be more relevant. However, we do not have the ability to reliably induce primary neurons undergo PS externalization. Furthermore, our goal is to demonstrate GPR56 can binds phosphatidylserine on live cells. In this regard, Ba/F3 cell line is well established cell-based model system (Gyobu et al., 2017; Miyanishi et al., 2007; Suzuki et al., 2010).

5. *Fig. 3: a functional characterization of microglia lacking GPR56 or GPR56 S4 would be essential. Is the general phagocytic activity of microglia impacted by the lack of either protein? Is the inflammatory profile changed?*

Response: Our *in vivo* engulfment assay showed that microglial GPR56 conditional ko (CKO) mice exhibited impaired phagocytosis of RGC inputs (See Fig. EV 5C and D). Interestingly, microglia lacking GPR56 didn't show significant defects in general cellular properties, like cellular density, %CD68, morphology (See Fig. EV 3C-J).

6. *Fig. 3I: The authors analyzed GPR56 S4 transcript in microglia at P14 in WT mice. Which brain area is microglia isolated from? Does GPR56 expression vary at P5, when microglia are actively pruning synapse in the dLGN, with respect to P13?*

Response: We thank the reviewer for the comments. We included experimental details in the method section in this revised manuscript. "Microglia were isolated from whole brains without cerebellum" (Page 25, line 11). Furthermore, we performed qPCR using P5 microglia, and showed that a steady increased of the transcript level from P5 to P14, which is consistent with previous reports (Fig. 3I, Appendix Fig. S1 and Bennet et al., PNAS 2016).

7. *Fig. 3 J-K: here the Authors, correctly, introduce the use of synaptic markers. However, defining the colocalizing Vglut1/homer1 structures as "synapse number" is not proper. The Authors should stick to the term "colocalizing Vglut1/homer1 puncta".*

Response: We agree with the reviewer's comment. In this revised manuscript, we specified colocalized vGlut2[+]/Homer1^{+} puncta as "retinogeniculate synapses" in the dLGN (Page 9, line 22), and vGlut1[+]/Homer1^{+} puncta as "corticogeniculate synapses" in the dLGN (Page 13, line 9).

8. *Fig. 3: The quantitation of synaptic puncta engulfed by microglia in GPR56 KO and S4 is mandatory, in order to state that "GPR56 S4 isoform is crucial to developmental synaptic refinement".*

Response: We thank the reviewer for the comment and have since performed the experiments and included our new data (Fig. 3M-O).

9. *Fig. 3: Also, although the Authors report that the increased synapse density is not the result of altered RGC number, given that GPR56 is involved in neuronal development, a quantitation of neuronal arborization should be performed in GPR56 KO and S4, to exclude that the differences in Vglut1/homer1 positive puncta depends on neuron-autonomous factors, different from synaptic pruning.*

Response: This request is reasonable in principle. Unfortunately, we found our inability to fulfill the request due to the following reasons:
1. Synapse number could alter without change in neuronal arborization. The synapses we analyzed are vGlut2$^+$ presynaptic inputs from RGCs. In early postnatal stage, the number of synapses decreases dramatically, but the average RGC arbor size and complexity remain largely unchanged (Hong et al., 2014). RGCs exhibit many *en passant* synapses (synapses on the stem of the RGC axons) that undergo elimination without changing the axonal structure (Hong et al., 2014).

2. Golgi staining will not work in this case, because it can't differentiate RGC arbors from dendritic arbors of local relay neurons and cortical neuronal arbors in the dLGN. Importantly, cortical inputs comprise ~90% synapses in dLGN.

3. It seems to us that generating RGC reporter line to visualize arbors as used in Hong et al., Eur J Neurosci 2019 would be an option. However, this will require up to 1-2 years of time to cross the reporter line into Gpr56 KO and S4 mutant background. Furthermore, there are over 20 different types of RGC cells, and different RGC classes exhibit distinct arborization patterns in the dLGN (Hong et al., 2019), and it is impossible for us to determine which type of RGC cells to study.

4. It is correct that GPR56 is expressed in first born neurons in the developing neocortex. However, it is largely absent in mature neurons. Furthermore, to address neuron-autonomous factors, we generated both microglial *Gpr56* constitutive conditional ko and microglial *Gpr56* tamoxifen-inducible ko mice, where neuronal *Gpr56* expression was not affected. We observed similar synapse defects as seen in *Gpr56* global ko mice, supporting that the synaptic chance was NOT due to "neuron-autonomous factors".

10. *Fig. 3: Also, based on the decrease in MBP in the corpus callosum, the possibility that demyelination might impact synaptic pruning by microglia should be at least discussed.*

Response: We thank the reviewer for the comment. We discussed it in the main text (please see Page 18, line 6-12).

11. *Fig. 3K and L: The images in Fig. 3K show an apparent higher density of Vglut2+ puncta in Gpr56 null mice, while Homer1+ puncta seem to be increased in both GPR56 null and GPR56 S4. A quantification of Vglut2 or Homer1 puncta, separately, could be of interest to investigate the possibility that GPR56 may be involved in mediating predominantly the elimination of presynaptic or postsynaptic puncta.*

Response: We quantified the densities of vGlut2$^+$ presynaptic inputs and homer1$^+$ postsynaptic signals, and only found increased vGlut2$^+$, but not homer1$^+$ in *Gpr56 null* (see new Appendix Figure S4, Page 10, line 1-2).

12. *Fig* S3 and Bennet et al., PNAS 2016, show that GPR56 transcript increases in microglia during development. The authors should discuss the meaning of this increase, since, according to their hypothesis, GPR56 is critical during the synaptic pruning period.

Response: We appreciate this comment. *Gpr56* transcript increases in microglia from embryonic stage and reaches a relatively high level between P3-P6, a period of active microglia-mediated synaptic pruning (Appendix Fig. S1, Page 7, line 1-2).

13. *Fig. 4: while this figure is consistent with the concept that "Deleting microglial GPR56 results in excess synapses in the dorsolateral geniculate nucleus during postnatal development", again no evidence is presented that the increased synaptic density is the consequence of a defect in synaptic pruning. Some experiments would be crucial to address this possibility: qPCR should be performed to demonstrate that the higher synaptic protein expression does not result from increased synaptic protein transcription in mutant mice. Even more importantly, analysis of synaptic engulfment by microglia should be performed by quantifying the synaptic markers internalized in microglial CD68-positive structures.*

Response: We appreciate this suggestion. However, we are constrained by technical limitations. Dorothy Schafer showed that most proteins engulfed by microglia will be quickly degraded once in lysosomes, which makes it unreliable to do synaptic engulfment analysis (Schafer et al., 2014). On the contrary, Alexa dye is more resistant to lysosomal hydrolases (Mukhopadhyay et al., 2010), making Alexa conjugated CTB as a robust dye to label engulfed material. Therefore, we performed our microglial engulfment assay using state of the art technology and showed a decreased engulfment in microglial GPR56 ko mice (Please see Fig. EV5C and D).

14. *Fig. 4 C and D: A quantification of Vglut2+ or Homer1+ puncta, separately, could be of interest (see above).*

Response: We included the quantification of Vglut2$^+$ or Homer1$^+$ puncta as a new Appendix Fig. S6.

15. *Fig. 3*: 4 E-H: Quantification by structured illumination microscopy (SIM) was performed in P8 mice brains, while WB analysis of CTR and CKO mice was performed at P30. The authors should add WB quantification of Vglut2 in the dLGN also at P8. Also, WB quantification of postsynaptic markers should be added, together with qPCR analyses for both pre and postsynaptic protein mRNAs.

Response: We performed the experiment as suggested and included the new data in this revised manuscript (Please see new Fig. 4 G-I, Page 12, line 6-9).

16. *Page 14: "Considering that PS binds GPR56 and flags synapses for removal by microglia" This sentence should be modified. The authors only showed that PSVue+ puncta colocalize with CTB-488. They did not perform staining with synaptic markers and omitted to do additional controls (see comments to Fig.1).*

Response: We appreciate the suggestion and modified the sentence as "Considering that PS binds GPR56 and flags RGC presynaptic inputs for removal by microglia" (Page 15, line 18).

17. *Fig. 5: this figure should be removed. A convincing demonstration that "microglial GPR56 regulates hippocampal synaptic development in a circuit-dependent manner" would require significantly deeper analyses (characterization of GPR56 expression in different hippocampal regions, quantitation of microglial engulfed synaptic material, Western Blotting, qPCR and so on).*

Response: In response to the reviewer's comment and editor's guidance, we have reworded our statement as "Microglial GPR56 regulates hippocampal synaptic development" (Page 13, line 14).

18. *Fig.* 7A-B: Please, see the comments above (Figure 1 C-D)

Response: As the same as Fig 1C-D, the measure was normalized to total number of RGC inputs.

19. *Fig. 8: the scheme provided is far ahead of the actual results reported in this manuscript.*

Response: We respectively disagree with this comment. Gratefully, we thank our editor Dr. Karin Dumstrei on her encouragement of including this figure.

20. *Page 6: Please provide the expanded definition for NTF. The figure legends 2 A and B should explain better how the authors engineered recombinant proteins.*

Response: We added the full name "N-terminal fragment" for NTF in the main text (Page 7, line 7). And in the Figure legend 2, we changed it to "(A) A schematic drawing of GPR56 protein structure, with a N-terminal fragment (NTF), a seven transmembrane domain (7-TM) and a C-terminal fragment (CTF). (B) A diagram shows the hFc tag was added to the c-terminal of GPR56-NTF (NTF-hFC) or GAIN domain (GAIN-hFc)." (Page 36, line 2-5)

21. *Fig. 4 A and B. Which area were analyzed? What is the mice's age? Please, specify this in the text and fig. legends.*

Response: We added the details of brain area and age in the main text: "We further performed RNAscope analysis for *Gpr56* in the prefrontal cortex of P30 mice" (Page 10, line 21-22). In the figure legends 4, we added "RNAscope was performed in the prefrontal cortex of P30 mice." (Page 39, line 5)

22. *Pag. 11: Please provide the expanded definition for Brn3a+*

Response: We added the definition for Bra3a+ in the main text. "brain-specific homeobox/POU domain protein 3A positive (Brn3a^{+}, a marker of RGC). (Page 12, line 10)

Reviewer #2:
1. *Using PSVue to label PS presynaptic elements or PS on BA/F3 cells, the authors show that the GAIN domain of GPR56 binds PS. For the in vivo synaptic pruning experiments, the authors use the widely used anterograde tracer cholera toxin B to show PS+ engulfment. Could the authors address if there are any confounders about CTB binding lipid rafts which presumably PSVue may also bind? For example, to show that PS+ terminals are indeed presynaptic phagocytosed elements, combining analysis of PS+ Homer+ elements within microglia?*

Response: We appreciated the reviewer for this thoughtful comment. To demonstrate the specificity of PSVue binding to synapses, we performed additional experiment, Iba1/CD68/vGlut2/PSVue co-staining, and showed PSVue[+]/vGlut2[+] retinogeniculate synapses colocalized with CD68, and phagocyted by microglia (Please see new Fig. 1H, Page 6, line 15). Considering the fact that CTB binds lipid rafts, we

would expect to observe a similar colocalization between P6 and P13 if PSVue also binds to lipid rafts. However, we observed that PSVue only colocalized with ~10% CTB at P6 and 3% at P13.

> 2. *It would be helpful to include a diagram as in Fig3A with the GPR56 NULL mouse for comparison.*

Response: We thank the reviewer for the thoughtful suggestion and included a diagram in Fig 3A in this revised manuscript.

> 3. *As the stated justification to use CX3CR1-Cre, this reviewer would recommend authors not necessarily use Zhao et al 2019 as proof that it is microglia specific, since the reasoning is less likely non-specific recombination of the reporter but rather different sensitivity of reporters; would instead recommend that authors emphasize their efforts to show microglia specific knockout.*

Response: We thank the reviewer for the suggestion. We removed the paper of Zhao et al 2019 and included the discussion on our generation of the microglial *Gpr56* specific knockout mice. (Page 10, paragraph 3).

> 4. *Using the RNAscope to show loss of GPR56 in microglia… is not, however, showing microglia-specificity, as an analysis of # non-microglial cells expressing GPR56 would be a more valid approach. Unless looking at recombination, however, would rephrase explanation that their approach unlikely non-specifically targeting cells in the retinogeniculate system given that GPR56 levels in non-microglial cells are comparable.*

Response: We appreciate reviewer's suggestion. In this revised manuscript, we included a new Fig. 4B and rephrased our discussion (Page 10, line 21-23; Page 11, line 1-2).

> 5. *Is the % increase in synaptic number observed for CKO for the various methods (Homer/VGlut1, SIM) comparable?*

Response: The % of increase was different but comparable between SIM and confocal data. Specifically, we observed ~35% increase in CKO at P10 using traditional confocal imaging, and a ~50% increase in CKO at P8 using SIM. The small difference could result from age differences (P10 vs P8), and/or from method differences (traditional confocal vs SIM).

> 6. *In the abstract, the authors state that developmental synaptic remodeling defects lead to neurodevelopmental disorders. This struck me as a bit absolute - not all neurodevelopmental are caused by synaptic pruning defects, and it is largely an implication that this process underlies neurodevelopmental disorders.. Rather than direct lines of proof. The authors write with a more balanced voice in the Concluding remarks and I would recommend they slightly edit the language in the abstract to respect this nuance.*

Response: We appreciate the comments and have since reworded "Developmental synaptic remodeling is important for the formation of precise neural circuitry and its disruption has been linked to neurodevelopmental disorders such as autism and schizophrenia." (Page 2, line 1-3).

Reference

Gyobu, S., Ishihara, K., Suzuki, J., Segawa, K., and Nagata, S. (2017). Characterization of the scrambling domain of the TMEM16 family. Proceedings of the National Academy of Sciences of the United States of America 114, 6274-6279.

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Suzuki, J., Umeda, M., Sims, P.J., and Nagata, S. (2010). Calcium-dependent phospholipid scrambling by TMEM16F. Nature 468, 834-838.

Dear Xian,

Thanks for sending me the revised manuscript. I have now had a chance to take a look at it and I appreciate the introduced changes. I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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The data shown in figures should satisfy the following conditions:

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➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
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 • definition of 'center values' as median or average;
 • definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ⬇ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size was got by running power analysis on the website of http://www.http://biomath.info/power/index.html. And our sample size was similar to that reported in recent other studies on a similar topic.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For comparing two groups of data, unpaired t-test between two groups was chosed on the website. After inputting standard deviation and difference between means of two groups, the estimated sample size will be outputted.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Animals were allocated into different groups (ie. Control vs. KO) by stratified randomination.
For animal studies, include a statement about randomization even if no randomization was used.	Phenotypes were compared between different genotype groups (ie. Control vs. KO). Within each genotype group, mice were selected randomly.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes, data were acquired and analyzed blindly.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Mice were encrypted and decoded by person #1. The experiments and analysis were carried out by person #2 blindly.
5. For every figure, are statistical tests justified as appropriate?	Yes. Student's t-test, one-way ANOVA, or two-way ANOVA were used appropriately.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. The data was assessed by Shapiro-Wilk normality test on the website of http://www.statskingdom.com/320ShapiroWilk.html.
Is there an estimate of variation within each group of data?	No.

Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	guinea pig anti-vGlut2, 1:1000, Millipore AB2251-I; guinea pig anti-vGlut1, 1:1000, Millipore AB5905; rabbit anti-Homer1, 1:250, Synaptic Systems, 160 003; rabbit anti-Iba1, 1:250, Wako 019-19741; guinea pig anti-Iba1, 1:500, Synaptic Systems 234 004; rat anti-CD68, 1:250, AbD Serotec MCA1957; rat anti-MBP, 1:100, Abcam ab7349
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	293T cells were obtained from ATCC and stored in liquid nitrogen for many years, and have not been tested for mycoplasma contamination recently. Ba/F3 cell line was a gift from Dr. Scott Manalis lab in MIT, and we're not sure whether it was recently authenticated and tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Gpr56fl/fl mice were generated as previously described (Giera et al., 2015). The Cx3Cr1-cre (B6J.B6N(Cg)-Cx3cr1tm1.1(cre)Jung/J, #025524) and Cx3Cr1-creER (B6.129P2(Cg)-Cx3cr1tm2.1(cre/ERT2)Litt/WganJ, #021160) mice were obtained from Jackson Laboratories. Considering both Cx3Cr1Cre and Cx3Cr1CreER are knock-in mice, we crossed these mice with Gpr56fl/fl to generate Gpr56fl/fl;Cx3Cr1-cre (or creER)+/- as conditional knockout mice, and Gpr56+/+; Cx3Cr1-cre (or creER)+/- as controls. CreER is a tamoxifen-inducible Cre recombinase. To induce a deletion of microglial Gpr56, 40 μg tamoxifen (in corn oil, Sigma) per day for 3 consecutive days (P1-P3) were given to neonatal animals via intraperitoneal injection (Parkhurst et al., 2013). To generate Gpr56 null mice, Gpr56fl/fl mice were crossed with CMV-cre mice (JAX stock #006054) (Schwenk et al., 1995) to delete exons 4-6, causing a deletion of Gpr56 in all tissues. RosaGFP reporter mice were made from pR26 CAG/GFP vector (Plasmid #74285, Addgene), which contains a loxP-flanked STOP cassette and a GFP reporter, which is expressed under the control of an IRES. Then the vector was micro-injected into C57BL/6 zygotes to make transgenic mice via CRISPR/Cas9 strategy in the Transgenic Core Laboratory of Boston Children's
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All the experiments involving mice were approved by Boston Children's Hospital and University of California, San Francisco.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm that all mice were handled according to the guidelines of Animal Care and Use Committee at Boston Children's Hospital and University of California, San Francisco, which meet the guideline of NIH.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA